Journal of Chemical Ecology, Vol. 30, No. 10, October 2004 (© 2004)

BIOACTIVITY OF LIPOPHILIC METABOLITES FROM GLANDULAR TRICHOMES OF *Medicago sativa* AGAINST THE POTATO LEAFHOPPER

CHRISTOPHER M. RANGER,[1,*] RUDOLPH E. K. WINTER,[2] GEORGE E. ROTTINGHAUS,[3] ELAINE A. BACKUS,[1,4] MARK R. ELLERSIECK,[5] and DAVID W. JOHNSON[6]

[1]*Department of Entomology and Interdisciplinary Plant Group*
1-87 Agriculture Building
University of Missouri, Columbia, Missouri 65211, USA

[2]*Department of Chemistry and Biochemistry*
University of Missouri, St. Louis
Missouri 63121, USA

[3]*Center for Phytonutrient and Phytochemical Studies, and Veterinary Medical Diagnostic Lab*
University of Missouri, Columbia, Missouri, 65211, USA

[4]*Current address: Exotic & Invasive Diseases & Pests Research*
USDA, ARS, PWA, Parlier
California 93648, USA

[5]*Department of Statistics, University of Missouri*
Columbia, Missouri 65211, USA

[6]*Cal/West Seeds*
N4505 County Hwy M West Salem
Wisconsin 54669, USA

(Received February 2, 2004; accepted June 15, 2004)

Abstract—*Medicago sativa* cv. G98A is highly resistant to the potato leafhopper, *Empoasca fabae*. Glandular trichome extracts from G98A were fractionated using flash chromatography and tested for settling deterrency against the potato leafhopper. A fraction of intermediate polarity exhibited strong, dose-dependent deterrency when applied to the surface of an artificial diet sachet. Deterrency was not detected, however, when the fraction was applied to the internal surface of the sachet membrane (i.e., when contact was limited to only the leafhoppers' stylets). Major components of the highly deterrent fraction, determined by gas chromatography – mass spectrometry, were a homologous series of fatty acid

* To whom correspondence should be addressed. Current address: Philip E. Marucci Center for Blueberry and Cranberry Research and Extension, Rutgers, The State University of New Jersey, Chatsworth, New Jersey 08019, USA. E-mail: ranger@aesop.rutgers.edu

0098-0331/04/1000-1969/0

amides $C_nH_{2n+1}NO$ ($n = 19$–23) and trace components were 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, and possibly 18:1 free fatty acids. Deterrency declined slightly, but was still strong, after fatty acids were removed from crude extracts. When the crude extracts were separated further, a fraction containing only the fatty acid amides was also deterrent. Activity increased when this fraction was supplemented with authentic (C12:0 through C18:0, and C18:1) free fatty acids. However, the authentic free fatty acids were not deterrent when tested without the lipophilic amides. Fatty acid amides and free fatty acids in trichomes of *M. sativa* G98A may synergize and together function in deterring settling by the potato leafhopper.

Key Words—Fatty acid amides, potato leafhopper, *Empoasca fabae*, alfalfa, *Medicago sativa*, host selection, glandular trichomes.

INTRODUCTION

The potato leafhopper, *Empoasca fabae* (Harris), is a generalist herbivore known to feed on over 200 host plants (Lamp et al., 1995). This insect is also considered to be the most significant pest of alfalfa, *Medicago sativa* L., in the midwestern and eastern United States. Cultivars of glandular-haired alfalfa expressing resistance to the potato leafhopper are currently commercially available. Consequently, *M. sativa* glandular trichomes have been the focus of recent studies, because of their presumed role in providing protection against the potato leafhopper (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower, 2001a,b, 2002; Shockley et al., 2002; Shockley and Backus, 2002).

Under field conditions and high leafhopper densities, resistant cultivars exhibit favorable growth characteristics, such as reduced leaf yellowing (i.e., hopperburn) and plant stunting (Lefko et al., 2000a,b; Sulc et al., 2001). Glandular-haired cultivars also have the ability to reduce field populations of potato leafhopper nymphs (Sulc et al., 2001). In addition, laboratory studies demonstrated that potato leafhopper development (Hogg and McCaslin, 1994), survivorship (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower, 2001b; Shockley et al., 2002), and host selection (Ranger and Hower, 2002; Shockley and Backus, 2002) were affected by the presence of *M. sativa* glandular trichomes.

Principally, the trichome-based resistance mechanism of glandular-haired *M. sativa* is associated with secretion of biologically active metabolites by erect and/or procumbent glandular trichome types (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower 2001a,b, 2002; Shockley et al., 2002). For instance, crude glandular trichome extracts from the resistant *M. sativa* genotype G98A deterred settling by potato leafhopper adults, and settling declined with an increase in extract concentration (Ranger et al., 2004). Fatty acid amides were major components of trichome extracts from *M. sativa* G98A (Ranger et al., 2004). These amides were not present in trichome extracts from less resistant glandular-haired *M. sativa* cv. G98C or from susceptible, nonglandular-haired *M. sativa*

cv. Ranger. Fatty acid amides are widespread in nature (Hannun et al., 1996) and have been isolated from a number of plants, grasses, and algae (Parmar et al., 1997; Faulds and Williamson, 1999; Kawasaki et al., 1998; Dembitsky et al., 2000). Here, we describe the fractionation and bioassay of major and some minor trichome components from *M. sativa* G98A.

METHODS AND MATERIALS

Insects and Plants. A colony of potato leafhoppers was maintained according to Hunter and Backus (1989). Insects were reared on greenhouse-grown fava beans *Vicia faba* (cv. "Windsor") using an environmental growth chamber (25 ± 2°C; 16:8 hr L:D). Cuttings (i.e., ramets) of resistant *M. sativa* genotype G98A were provided by Cal/West Seeds (West Salem, WI). Plants were vegetatively propagated and grown under greenhouse conditions. Metal halide lamps (400-W high-pressure sodium, were used to supplement the natural lighting. Experimental plants were about 4 mo old and harvested three times prior to use. In addition, extracts used in experiments were obtained from plants allowed to grow for about 25 days since last cutting.

Trichome Extraction and Fractionation. Trichomes were isolated from 150 g (fresh weight) of *M. sativa* G98A stem sections according to a modified protocol of Yerger et al. (1992) and described in detail by Ranger et al. (2004). In short, entire stems were harvested from 11:00 A.M. to 3:00 P.M. and immediately cut into 3-cm sections. About 1–2 g was transferred into a test tube and lowered into a Dewar flask containing liquid nitrogen (N_2). After submersion, the test tube was raised out of N_2 and vortexed for 3–5 sec, resulting in the freed trichomes adhering to the test tube walls. Isolated trichomes were rinsed and soaked in ethanol for 24 hr, along with 10 g Na_2SO_4 (as an overnight drying agent). Extracts were filtered through glass fiber filter paper (G6, Fisher Scientific, Pittsburgh, PA) and evaporated under reduced pressure using a rotary evaporator. Residues (crude weight: 17 mg) were redissolved in 2 ml of methylene chloride for fractionation.

Crude trichome extracts from *M. sativa* G98A were fractionated using flash chromatography (Still et al., 1978) (0.04–0.063 mm, 230–400 silica gel) with elution with methylene chloride containing increasing concentrations of ethanol. Four fractions were collected using the following elution concentrations: fraction 1 (F1) 100% methylene chloride; fraction 2 (F2) 3% ethanol in methylene chloride; fraction 3 (F3) 6% ethanol in methylene chloride; and fraction 4 (F4) 25–100% ethanol. Fractions were evaporated to dryness and residues of each fraction were redissolved in methylene chloride for gas chromatography – mass spectrometry (GC–MS) analysis or 2 ml of acetone for bioassay.

Preparation of Neutral and Acidic Extracts. The role of neutral and acidic compounds in deterring leafhopper settling was assessed by first isolating

trichomes from 150 g of G98A stem sections and extracting with ethanol. Crude extracts (with acidic compounds present) were concentrated to dryness using a rotary evaporator, and residues (crude weight: 17 mg) were redissolved in 2 ml of acetone for bioassay.

After testing crude trichome extracts with acidic compounds present, extracts were next concentrated to dryness and redissolved in 2 ml of methylene chloride. Acidic compounds were removed from the extracts by eluting through a column of Alumina (basic Grade I, Merck Co.) using 6% ethanol in methylene chloride. The resulting neutral eluants were concentrated to dryness by using a rotary evaporator, and residues (3.5 mg) were redissolved in 2 ml of acetone for bioassay.

After bioassay, the acid-free extracts were concentrated to dryness, redissolved in 2 ml of methylene chloride, and fractionated with flash chromatography (using the aforementioned procedures). The neutral fraction collected with 6% ethanol in methylene chloride was concentrated to dryness and residues (2.9 mg) were redissolved in 2 ml of acetone for bioassay.

A blend of authentic fatty acids (C12:0 through C18:0, and C18:1) was added to the neutral fraction. The mixture was added to the neutral fraction to represent, on the basis of GC–MS analysis, 10% of the total concentration of lipophilic amides present in the sample (2.9 mg). The total percent contribution of each free fatty acid to the stock mixture were as follows: 12:0 (5%), 13:0 (5%), 14:0 (10%), 15:0 (5%), 16:0 (54%), 17:0 (5%), 18:0 (4%), and 18:1 (12%). Finally, to test the free fatty acids alone, the same fatty acid blend was added to an acetone stock at the same concentration as was added to the neutral fraction (i.e., 10% of the amides).

Insect Acclimation for Bioassays. Prior to each bioassay, <5 day-old adult females were selected from the laboratory fava bean colony and transferred to a cage containing two mature *M. sativa* Ranger plants for a 24 hr period of conditioning. Insects were then transferred to a sachet containing artificial diet for an additional 24 hr of acclimation. Acclimation sachets were based on a design by Habibi et al. (1993) and described in detail by Ranger et al. (2004). In short, acclimation sachets were prepared by stretching Parafilm® over a disc of solidified artificial diet (approximately 8.0-cm diameter, 1.0 cm high). Sachets were placed Parafilm® side up in the bottom of petri dish (8.5-cm diameter), and a petri dish lid was used to contain the leafhoppers. Artificial diet consisted of an aqueous solution of 5% (wt:vol) sucrose and 4% (wt:vol) low-melt agarose (Sea Plaque Agarose, FMC Inc., Rockland, ME).

Two-Choice Bioassays of Fractionated Extracts. Fractionated trichome extracts were bioassayed using a feeding sachet based on a design by Habibi et al. (1993) and described in detail by Ranger et al. (2004). In short, sachets were prepared by pipetting artificial diet into a single plastic gasket (1×1 cm^2) positioned on a microscope cover slip. Parafilm® was stretched over the gasket and trimmed

back to within the gasket edges. Completed sachets were stored at 1°C for 24 hr prior to their use in bioassays.

A micropipette was used to apply aliquots from stock solutions to the exposed Parafilm® surface of the sachet. The acetone was allowed to evaporate from the sachet surface under ambient conditions for about 25 min. Each of the four fractions were tested at concentrations representing 7.5, 3.75, and 1.88% of the initial stock solution. In all experiments, control sachets were prepared by applying aliquots from an acetone stock (using the corresponding volume for a particular comparison).

After fractions were applied to sachets, two-choice comparisons were made by placing a treated and control sachet at opposing regions within test arenas, which consisted of a clear plastic tube (7.0 cm diameter, and 3.5 cm in high) positioned upright in a Petri dish lid. Once sachets were situated, a Petri dish lid was used to seal the test arena, and 10 acclimated leafhoppers were aspirated into each arena. Test arenas were arranged in a completely randomized design in an aluminum tray under constant fluorescent light, and water was added to the bottom of the tray to prevent desiccation. After 60 min of acclimation, the number of insects settling/feeding on a particular diet surface was recorded at 15 min intervals over the next 350 min.

Numbers of leafhoppers settling on each sachet were converted into proportions and arcsine square root-transformed. Data were analyzed with the SAS general linear model (GLM) procedure (SAS Institute, 1985) using a repeated measures split plot analysis of variance (ANOVA). Treatment was used as the main plot effect in the linear statistical model for comparisons of settling. The subplot contained the effects of Time and Treatment × Time. If a significant Treatment × Time interaction was present, then differences between means for ANOVAs were compared with least significant difference (SAS Institute, 1985).

Site of Activity. To determine the portion of the potato leafhopper's feeding apparatus on which the compounds in F3 act, a modified two-choice bioassay was conducted whereby 1.88% of the 2 ml F3 solution was first applied to the Parafilm® surface and the solvent was allowed to evaporate. The piece of Parafilm® was then inverted before it was laid over the artificial diet, resulting in the treated surface coming in direct contact with the diet. The Parafilm® surface exposed to the labium and the rest of the body was consequently untreated, thereby limiting contact with F3 to only the leafhoppers' stylets. Control sachets were prepared using the corresponding volume of acetone. Treated and control sachets were stored at 1°C for 24 hr, to allow the extract to diffuse partially. After this time, sachets were tested for settling deterrency using the previously described two-choice bioassay. Data were transformed and analyzed using the aforementioned procedures.

Effects of Acid Removal and Addition on Activity. Crude extracts with acidic compounds present and absent were bioassayed at 1.88% of the initial stock

solution, using two-choice design. A neutral fraction with composition similar to F3 was also tested at 1.88% of the initial stock, and also after being supplemented with authentic free fatty acids. The same blend of authentic fatty acids was also tested alone at 1.88% of the stock. In each experiment, numbers of leafhoppers settling on trichome extracts were compared with numbers settling on a solvent control.

GC–MS Analysis. Crude and fractionated extracts redissolved in methylene chloride were analyzed using an Hewlett-Packard 5890 gas chromatograph equipped with a mass spectra detector operating in electron impact (EI) mode (70 eV). A Restek Rtx-1 column (15 m × 0.25 mm × 0.3 μm) or equivalent was used. The injector port was held at 250°C, and the oven was programmed from 100 to 320°C at 10°C/min and held at 320°C for 5 min.

RESULTS

Two-Choice Bioassays of Fractionated Extracts. At 7.5% of the initial stock solution, or 488 μg/cm^2, the third fraction (F3) collected by flash chromatography of *M. sativa* G98A trichome extracts possessed the most significant degree of deterrency in two-choice bioassays ($F = 73.04$; $df = 1$; $P < 0.001$; Figure 1C). This fraction weighed 6.5 mg and represented about 38% of the total material recovered, compared to 5 mg for F1, 2.5 mg for F2, and 3.0 mg for F4. At 7.5% of the initial stock, or 225 μg/cm^2, F4 provided the only other deterrency, but it was not highly active ($F = 5.30$; $df = 1$; $P = 0.044$; Figure 1D). Similarly, the number of leafhoppers settling on F2 was only marginally less than those on control sachets when tested at 188 μg/cm^2 ($F = 4.43$; $df = 1$; $P = 0.059$; Figure 1B). F1 did not significantly ($P > 0.05$) deter leafhopper settling compared against control sachets at 375 μg/cm^2 (Figure 1A). A Treatment × Time interaction was not detected ($P > 0.05$) for any of the fractions tested at 7.5% of the initial stock solution.

F3 was also highly deterrent to leafhopper settling when tested against control sachets at 3.75% of the initial stock solution, or 244 μg/cm^2 ($F = 41.21$; $df = 1$; $P < 0.001$; Figure 2C). A Treatment × Time interaction was detected for F4 at 3.75% of the initial stock solution, or 115 μg/cm^2 ($F = 2.47$; $df = 18$; $P < 0.001$; Figure 2D). Control sachets were not significantly ($P > 0.05$) preferred over F1 at 188 μg/cm^2 (Figure 2A) or F2 at 94 μg/cm^2 (Figure 2B). At 3.75% of the initial stock solution, a Treatment × Time interaction was not detected ($P > 0.05$) for F1, F2, or F3.

Activity slightly declined with a decrease in concentration, but F3 remained highly active at 1.88% of the initial stock solution, or 122 μg/cm^2 ($F = 33.30$; $df = 1$; $P < 0.001$; Figure 3C). F2 at 47 μg/cm^2 did not significantly ($P > 0.05$) affect selection behavior of the potato leafhopper (Figures 3B). A Treatment

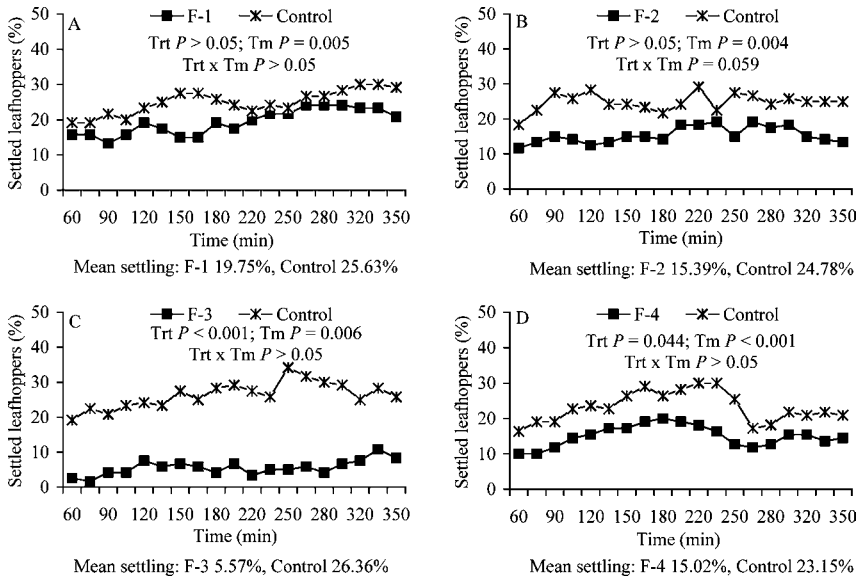

FIG. 1. (A–D). Two-choice bioassay of potato leafhoppers for settling on fractions from *M. sativa* G98A glandular trichomes or a solvent control. Fractions were applied to the surface of an artificial diet sachet at a concentration of 7.5% of the initial stock solution, which represented 375 μg/cm^2 for F1 (A), 188 μg/cm^2 for F2 (B), 488 μg/cm^2 for F3 (C), and 225 μg/cm^2 for F4 (D). $N = 12$ for (A), (B), and (C), and $N = 11$ for (D). Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

$\times$ Time interaction was detected for F4 at 1.88% of the initial stock solution, or 56 μg/cm^2 ($F = 2.22$; $df = 18$; $P = 0.003$; Figure 3D). F1 was significantly preferred to control sachets at 1.88% of the initial stock, or 94 μg/cm^2 ($F = 6.22$; $df = 1$; $P = 0.03$; Figure 3A). At 1.88% of the initial stock solution, a Treatment $\times$ Time interaction was not detected ($P > 0.05$) for F1, F2, or F3.

Site of Activity. When F3 was applied at 1.88% of the initial stock solution, or 122 μg/cm^2, to the Parafilm® sachet's inner surface, the number of insects settling on treated sachets did not differ ($P > 0.05$) from control sachets (Figure 4). Therefore, leafhoppers were not deterred from settling when contact with F3 was limited only to their stylets.

GC–MS Analysis of Trichome Fractions. Major components of the highly active F3 were a homologous series of fatty acid amides $C_nH_{2n+1}NO$ ($n =$ 19–23), which have been previously characterized by electron impact and fast atom bombardment mass spectrometry (Ranger et al., 2004). The compounds are *N*-(3-methylbutyl)amides of straight chain C14:0 through C18:0 fatty acids, as

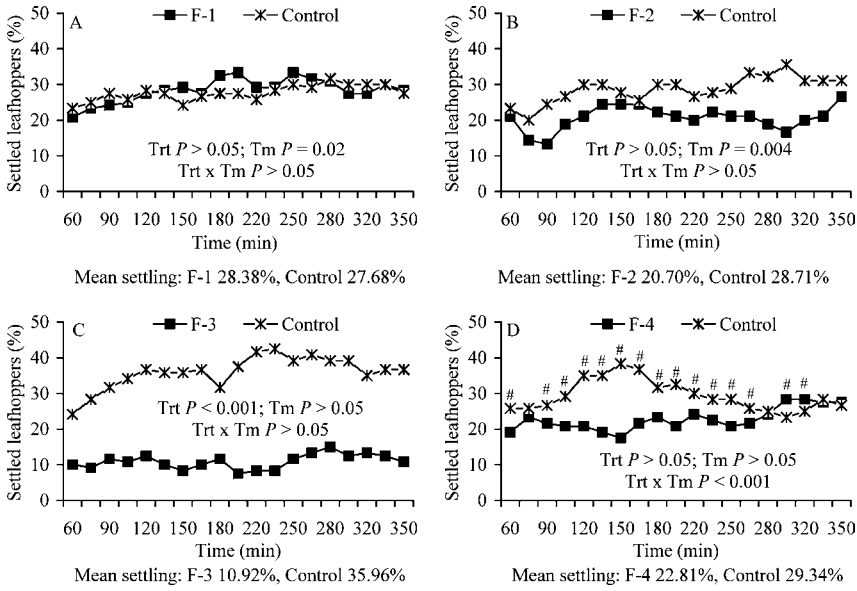

FIG. 2. (A–D). Two-choice bioassay of potato leafhoppers for settling on fractions from *M. sativa* G98A glandular trichomes or a solvent control. Fractions were applied to the surface of an artificial diet sachet at a concentration of 3.75% of the initial stock solution, which represented 188 μg/cm^2 for F1 (A), 94 μg/cm^2 for F2 (B), 244 μg/cm^2 for F3 (C), and 115 μg/cm^2 for F4 (D). Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details). Since a significant Treatment $\times$ Time interaction was associated with (D), any difference between the percentage of settled leafhoppers greater than an LSD value of 2.5% are significantly different ($P > 0.05$) and are distinguished by #. $N = 12$ for (A) and (D) and $N = 9$ for (B) and (C).

determined by comparison of mass spectra and retention times with those of synthetic representatives (Ranger, unpublished data). The fatty acid amides were in greatest concentration in F3, which was collected by eluting with 6% ethanol in methylene chloride. Trace components in the deterrent F3 were a homologous series of saturated free fatty acids $C_nH_{2n}O_2$ ($n = 12$–18), and an unsaturated C18 acid, likely oleic acid. Fatty acids and some of the amides were also present in lower concentration in the less active F4 fraction (Figure 1D). Similarly, trace amounts of the amides and fatty acids were present in F2, but this fraction was not deterrent at the concentrations tested.

Major components of F1, which was preferred to control sachets at 1.88% of the initial stock, or 93.75 μg/cm^2 (Figure 3A), were hydrocarbons and/or related alcohols, collected using 100% methylene chloride. Fatty acid amides and free fatty acids were not present in F1.

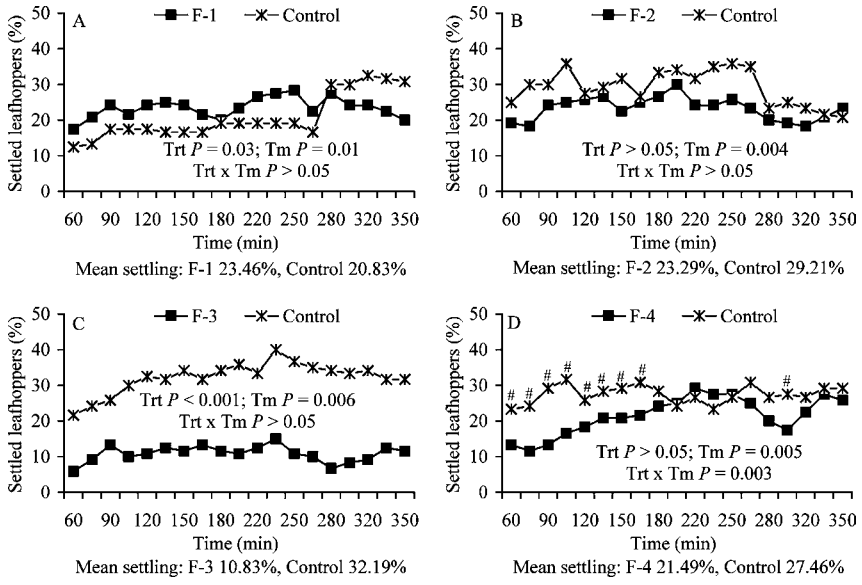

FIG. 3. (A–D). Two-choice bioassay of potato leafhoppers for settling on fractions from *M. sativa* G98A glandular trichomes or a solvent control. Fractions were applied to the surface of an artificial diet sachet at a concentration of 1.88% of the initial stock solution, which represented 94 μg/cm^2 for F1 (A), 47 μg/cm^2 for F2 (B), 122 μg/cm^2 for F3 (C), and 56 μg/cm^2 for F4 (D). Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details). Since a significant Treatment × Time interaction was associated with (D), any difference between the percentage of settled leafhoppers greater than an LSD value of 7.8% for (D) are significantly different ($P > 0.05$) and are distinguished by #. $N = 12$ for each comparison.

Effects of Acid Removal and Addition on Activity. Control sachets were significantly preferred to sachets treated with crude extracts ($F = 70.07$; $df = 1$; $P < 0.001$; Figure 5A). GC–MS confirmed the extracts contained lipophilic amides, free fatty acids, and linear hydrocarbons (and/or related alcohols).

The neutral extracts, when tested at 1.88% of the stock solution, or 66 μg/cm^2, were less deterrent ($F = 21.15$; $df = 1$; $P < 0.001$; Figure 5B). GC–MS confirmed the absence of free fatty acids, and the presence of fatty acid amides and hydrocarbons (and/or related alcohols) in the neutral extracts. At 1.88% of the initial stock, or 54 μg/cm^2, the amide-containing fraction remained deterrent against leafhopper settling ($F = 30.11$; $df = 1$; $P < 0.001$; Figure 5C). Activity did not appear to decline with loss of the hydrocarbons and/or related alcohols (compare Figure 5B and C).

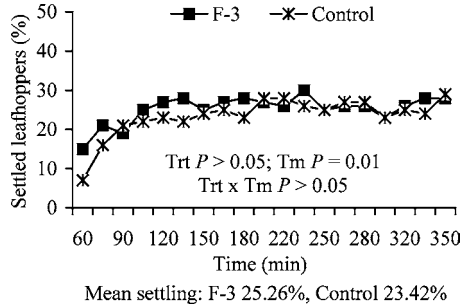

FIG. 4. Deterrency of fraction F3 from *M. sativa* G98A trichome extracts when contact was limited to the potato leafhoppers' stylets. Leafhoppers were offered a choice between sachets treated with F3 or a solvent control ($N = 12$). F3 was applied to the surface of an artificial diet sachet at a concentration of 1.88% of the initial stock solution, which represented 122 mg/cm^2. Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

To test for synergism, when free fatty acids were added to the amide-containing fraction and tested at 1.88% of the initial stock, or 59.5 μg/cm^2, the extracts became highly deterrent to leafhopper settling ($F = 115.50$; $df = 1$; $P < 0.001$; Figure 5D). However, at 1.88% of the stock, or 5.45 μg/cm^2, the blend of authentic free fatty acids alone was not deterrent against leafhopper settling ($P > 0.05$; Figure 6).

DISCUSSION

The presence of saturated fatty acid amides predominantly in the highly deterrent F3 suggests that these compounds possess biological activity. Lipophilic amides were the only GC–MS detectable compounds in a deterrent neutral fraction (Figure 5C) after free fatty acids were selectively removed. Fatty acid amides have not been well documented to affect insect behavior, but isobutylamides have long been known to possess insecticidal activities (Elliot et al., 1987). For instance, plant-derived isobutylamides of unsaturated, aliphatic, straight chain C10–C18 acids are toxic to a variety of insects (Jacobson, 1971).

Saturated (C12:0 through C18:0) and possibly unsaturated (18:1) free fatty acids in the deterrent F3 also suggest that these compounds assist in deterring leafhopper settling. Deterrency decreased when fatty acids were removed from crude *M. sativa* G98A trichome extracts (compare Figure 5A and B). While this decline in activity may be explained by partial loss of active compounds after elution through basic Alumina, supplementation of the amide-containing fraction with authentic fatty acids (C12:0–C18:0, and C18:1) resulted in increased

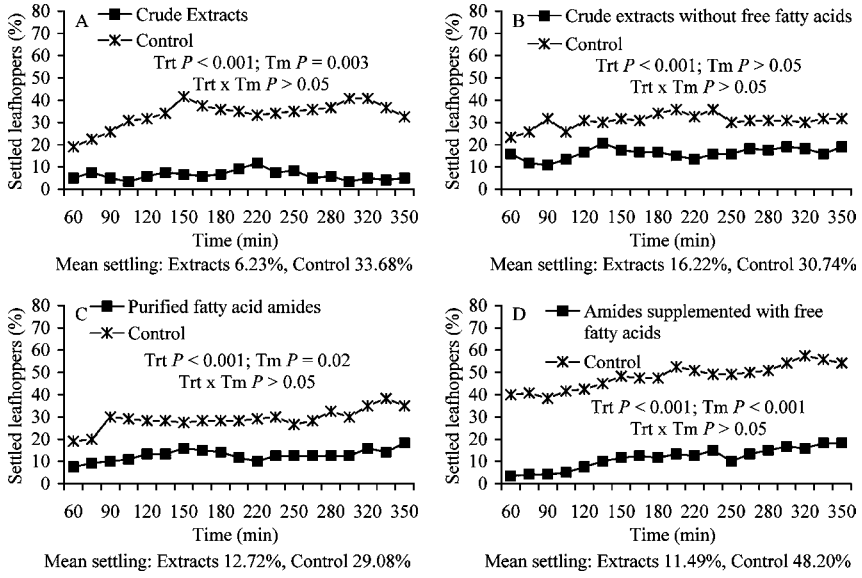

FIG. 5. (A–D). Activity of crude *M. sativa* G98A glandular trichome extracts with acidic compounds present (A) was compared against a solvent control. Acidic compounds were removed from the crude extracts (B) and bioassayed. Next, an amide-containing fraction (C) was isolated from the neutral, crude extracts, and tested for activity. The neutral, amide-containing fraction was supplemented with a mixture of authentic C12:0 through C18:0, and C18:1, free fatty acids (D). All extracts were tested at 1.88% of the initial stock solution, which represented 319, 66, 54, and 59.5 μg/cm^2 for (A), (B), (C), and (D), respectively. $N = 12$ for each comparison. Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

deterrency (Figure 5D). In fact, the degree of deterrency appeared to return to that of the crude extracts (compare Figure 5A and D). However, when tested alone at 5.45 μg/cm^2, the mixture of free fatty acids alone was not deterrent. These results suggest that C12–C18 free fatty acids may function in a synergistic fashion with the lipophilic amides, possibly by serving as a "carrier" for the deterrent amides. Synthetic representatives of the fatty acid amides and free fatty acids from *M. sativa* G98A glandular trichomes are currently being bioassayed to more completely understand the activity and interactions among these compounds. Settling by the green peach aphid, *Myzus persicae* Sulzer, is deterred by C8–C13 fatty acids at concentrations ranging from 1 to 100 μg/cm^2 (Greenway et al., 1978). Furthermore, longer chain free fatty acids (>C16) make up a large proportion of the surface lipids of some plants, but their effect on insect behavior is not well known (Eigenbrode and Espelie, 1995).

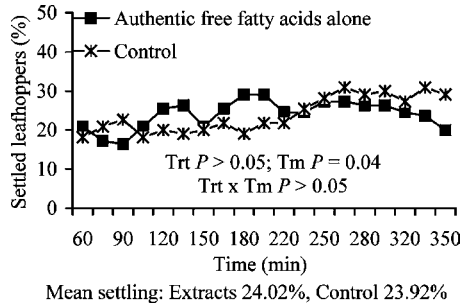

FIG. 6. Two-choice bioassay of potato leafhoppers for settling on artificial diet sachets treated with a mixture of C12:0 through C18:0, and C18:1, free fatty acids or a solvent control. The fatty acid mixture was tested at 1.88% of the initial stock solution, or 5.45 $\mu g/cm^2 (N = 11)$. Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

Since the piercing–sucking potato leafhopper is not ingesting the glandular trichomes, as would a chewing insect, it seems the deterrence of *M. sativa* G98A trichome extracts is a function of contact with the labium or other nonstylet part(s) of the body. The lack of deterrency observed when contact with the highly deterrent F3 was limited to only the leafhoppers' stylets (Figure 4) suggests that uptake to precibarial sensilla or the gut may not be necessary for deterrence. Similarly, sucrose esters of carboxylic acids from glandular trichomes of *Solanum berthaultii* Hawkes were not effective in deterring settling or probing by the green peach aphid when contact was limited to only the stylets (Neal et al., 1990). Chapman (1995) noted that Hemiptera lack chemoreceptors on the stylets, and while they may be present on the labrum and labium, they are used to detect chemicals on the external surface of the host (Backus, 1988; Chapman, 1995). However, it is possible that ingestion of alfalfa trichome metabolites by the potato leafhopper acts in conjunction with external stimuli (affecting the exoskeleton or contact chemoreceptors) to cause deterrence.

When stationary on the stem surface, the potato leafhopper's exoskeleton often comes in direct contact with secretory gland heads of the erect trichome type (Ranger, personal observation). Following contact, the lipophilic trichome metabolites might be absorbed through the cuticle and affect the potato leafhopper's physiology. Compared to the erect glandular trichome, however, the morphology of the procumbent trichome type appears better suited for positioning an exudate in such a manner to encounter the leafhopper's tarsal chemoreceptors. In essence, contact between the leafhopper's tarsi and secretions from the procumbent glandular trichome seems likely because the procumbent trichome is characterized by a bent stalk, which results in the gland head being in close proximity to the surrounding epidermal surface (Kreitner and Sorensen, 1979a; Ranger and

Hower, 2001a). Exudate released from the procumbent trichome then coats the epidermal surface near the collapsed gland head (Kreitner and Sorensen, 1979b; Ranger and Hower 2001a) and has even been observed to adhere to the potato leafhopper's tibia (Ranger and Hower, 2001a). Following contact with the contact chemoreceptors, trichome metabolites such as the fatty acid amides may then be recognized as deterrents. Alternatively, sensitivity of the tarsal chemoreceptors to phagostimulants may be reduced or impaired by the trichome metabolites. Potato leafhopper nymphs clean their tarsi using an excretory droplet significantly more often following contact with glandular trichomes on the resistant *M. sativa* cv. FGplh13 compared to nonglandular trichomes on a susceptible variety (Ranger and Hower, 2002). Such a behavioral response indicates recognition on part of the potato leafhopper to either the adhesive exudate or compounds present within the secretion.

Interestingly, F1 was actually attractive to the leafhopper when tested at 94 μg/cm^2 (Figure 3A). Linear hydrocarbons and/or related alcohols were the main components of this nonpolar fraction and may have been responsible for increased settling. A hydrocarbon fraction of *n*-alkanes (C27, C29, C31, and C33) from broad bean, *Vicia faba* L. stimulated feeding by the pea aphid, *Acyrthosiphon pisum* (Harris) (Klingauf et al., 1971, 1978). Additional studies are also needed to characterize the effects of hydrocarbons from *M. sativa* G98A on host selection by the potato leafhopper.

Overall, fatty acid amides and free fatty acids in glandular trichomes of *M. sativa* G98A appear to play a role in deterring settling by the potato leafhopper. Identification of natural products from glandular-haired *M. sativa* with activity against the potato leafhopper has significant implications for determining the chemical and molecular mechanisms by which resistance operates. The ability to screen for plants expressing high concentrations of deterrent compounds could increase the efficiency by which improved cultivars are selected.

Acknowledgments—We thank Cal/West Seeds (West Salem, WI) for providing the resistant and susceptible plant materials. We also thank Sanford Eigenbrode (University of Idaho) for critically reviewing this manuscript. This research was supported in part by a research gift from Cal/West Seeds and a University of Missouri Life Sciences Predoctoral Fellowship. This paper is a contribution from the Missouri Agricultural Experiment Station, Project number PSSL077.

REFERENCES

BACKUS, E. A. 1988. Sensory systems and behaviors which mediate hemiptera plant-feeding: A taxonomic overview. *J. Insect Physiol*. 34:151–165.

CHAPMAN, R. F. 1995. Chemosensory regulation of feeding, pp. 101–136, *in* R. Chapman, and G. de Boer (eds.). Regulatory Mechanisms in Insect Feeding. Chapman and Hall, New York.

DEMBITSKY, V. M., SHKROB, I., and ROZENTSVET, O. A. 2000. Fatty acid amides from freshwater green alga *Rhizoclonium hieroglyphicum*. *Phytochemistry*. 54:965–967.

EIGENBRODE, S. D. and ESPELIE, K. E. 1995. Effects of plant epicuticular lipids on insect herbivores. *Annu. Rev. Entomol.* 40:171–194.

ELDEN, T. C. and MCCASLIN, M. 1997. Potato leafhopper (Homoptera: Cicadellidae) resistance in perennial glandular-haired alfalfa clones. *J. Econ. Entom.* 90:842–847.

ELLIOT, M., FARNHAM, A. W., JANES, N. F., JOHNSON, D. M., and PULMAN, D. A. 1987. Synthesis and insecticidal activity of lipophilic amides. Part 1: Introductory survey, and discovery of an active synthetic compound. *Pestic. Sci.* 18:191–201.

FAULDS, C. B. and WILLIAMSON, G. 1999. The role of hydroxycinnamates in the plant cell wall. *J. Sci. Food Agric.* 79:393–395.

GREENWAY, A. R., GRIFFITHS, D. C., and LLOYD, S. L. 1978. Response of *Myzus persicae* to components of aphid extracts and to carboxylic acids. *Entomol. Exp. Appl.* 24:369–374.

HABIBI, J., BACKUS, E. A., and CZAPLA, T. H. 1993. Plant lectins affect survival of the potato leafhopper (Homoptera: Cicadellidae). *J. Econ. Entom.* 86:945–951.

HANNUN, Y. A., OBEID, L. M., and DBAIBO, G. S. 1996. Ceramide: A novel second messenger and lipid mediator, pp. 177–204, *in* R. M. Bell (ed.), Handbook of Lipid Research. Plenum Press, New York.

HOGG, D. B. and MCCASLIN, M. 1994. Performance of potato leafhopper nymphs and adults on selected clones of glandular-haired alfalfa, p. 62, *in* Report of the Thirty-Fourth North American Alfalfa Improvement Conference, July 10–14, Guelph, ON.

HUNTER, W. B. and BACKUS, E. A. 1989. Mesophyll-feeding by the potato leafhopper, *Empoasca fabae* (Homoptera: Cicadellidae): Results from electronic monitoring and thin-layer chromatography. *Environ. Entom.* 18:465–472.

JACOBSON, M. 1971. The unsaturated isobutylamides, pp. 137–176, *in* M. Jacobson and D. G. Crosby (eds.) Naturally Occurring Insecticides. Marcel Dekker, New York.

KAWASAKI, W., MATSUI, K., AKAKABE, Y., ITAI, N., and KAJIWARA, T. 1998. Volatiles from *Zostera marina*. *Phytochemistry* 47:27–29.

KLINGAUF, F., NÖCKER-WENZEL, K., and KLEIN, W. 1971. Einfluss einiger Wachskomponenten von *Vicia faba* L. auf das Wirtswahlverhalten von *Acyrthosiphon pisum* (Harris) (Homoptera: Aphidae). *Z. Pflanzenkr. Pflanzensch.* 78:641–648.

KLINGAUF, F., NÖCKER-WENZEL, K., and RÖTTGER, U. 1978. Die rolle peripherer Pflanzenwachse fur den Befall durch phytophage Insekten. *Z. Pflanzenkr. Pflanzensch.* 85:228–237.

KREITNER, G. L. and SORENSEN, E. L. 1979a. Glandular trichomes on *Medicago* species. *Crop Sci.* 19:380–384.

KREITNER G. L., and SORENSEN, E. L. 1979b. Glandular secretory system of alfalfa species. *Crop Sci.* 19:499–501.

LAMP, W. O., NIELSON, G. R., and DANIELSON, S. D. 1995. Patterns among host plants of the potato leafhopper, Empoasca fabae (Homoptera: Cicadellidae). *J. Kansas Entomol. Soc.* 67:354–368.

LEFKO, S. A., PEDIGO, L. P., and RICE, M. E. 2000a. Symptoms and growth of potato leafhopper-tolerant alfalfa in response to potato leafhopper feeding. *Agron. J.* 92:721–725.

LEFKO, S. A., PEDIGO, L. P., and RICE, M. E. 2000b. Alfalfa stand tolerance to the potato leafhopper and its effects on the economic injury level. *Agron. J.* 92:726–732.

NEAL, J. J., TINGEY, W. M., and STEFFENS, J. C. 1990. Sucrose esters of carboxylic acids in glandular trichomes of *Solanum berthaultii* deter settling and probing by green peach aphid. *J. Chem. Ecol.* 16:487–497.

PARMAR, V. S., JAIN, S. C., BISHT, K. S., JAIN, R., POONAM, T., JHA, A., TYAGI, O. D., PRASAD, A. K., WENGEL, J., OLSEN, C. E., and BOLL, P. M. 1997. Phytochemistry of the genus *Piper*. *Phytochemistry* 46:597–673.

RANGER, C. M., BACKUS, E. A., WINTER, R. E. K., ROTTINGHAUS, G. E., ELLERSIECK, M. R., and JOHNSON, D. W. 2004. Glandular trichome extracts from *Medicago sativa* deter settling by the potato leafhopper, *Empoasca fabae*. *J. Chem. Ecol.* 30:921–937.

RANGER, C. M. and HOWER, A. A. 2001a. Glandular morphology from a perennial alfalfa clone resistant to the potato leafhopper. *Crop Sci*. 41:1427–1434.

RANGER, C. M. and HOWER, A. A. 2001b. Role of the glandular trichomes in resistance of perennial alfalfa to the potato leafhopper (Homoptera: Cicadellidae). *J. Econ. Entom*. 94:951–957.

RANGER, C. M. and HOWER, A. A. 2002. Glandular trichomes on perennial alfalfa affect host-selection behavior of *Empoasca fabae*. *Ent. Exp. Appl*. 105:71–81.

SAS INSTITUTE. 1985. Statistical Analysis System. SAS Institute, Cary, NC.

SHOCKLEY, F. W. and BACKUS, E. A. 2002. Repellency to the potato leafhopper (Homoptera: Cicadellidae) by erect glandular trichomes on alfalfa. *Environ. Entom.* 31:22–29.

SHOCKLEY, F. W., BACKUS, E. A., ELLERSIECK, M. R., JOHNSON, D. W., and MCCASLIN, M. 2002. Glandular-haired alfalfa resistance to potato leafhopper (Homoptera: Cicadellidae) and hopperburn: Development of resistance indices. *J. Econ. Entom*. 95:437–447.

STILL, C. W., KAHN, W., and MITRA, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43:2923–2925.

SULC, R. M., VAN SANTEN, E., JOHNSON, K. D., SHEAFFER, C. C., UNDERSANDER, D. J., BLEDSOE, L. L., HOGG, D. B., and WILSON, H. R. 2001. Glandular-haired cultivars reduce potato leafhopper damage in alfalfa. *Agron. J*. 93:1287–1296.

YERGER, E. H., GRAZZINI, R. A., HESK, D., COX-FOSTER, D. L., CRAIG, R., and MUMMA, R. O. 1992. A rapid method for isolating glandular trichomes. *Plant Physiol*. 99:1–7.